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RELATION OF CATALASE, OXIDASE, AND H^+ CONCENTRATION TO THE FORMATION OF OVERGROWTHS

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Overgrowths were found by the author (7) to result from local freezing of the leaf tissue in a number of plants, including cabbage (*Brassica oleracea capitata*) and *Bryophyllum calycinum*. These overgrowths offer special opportunity for the separation of certain factors concerned in the rejuvenescence of cells, and for the comparison of physiological conditions attendant upon the production of overgrowths resulting from infection with *Bacterium tumefaciens* and from the stimulation of normal tissue by physical and chemical means. The literature of overgrowths as a result of bacterial infections in plants has been fully presented in papers by Dr. Erwin F. Smith.

The economic importance (9, 19, 20) of certain plant diseases in which overgrowths are produced, and the relation of these to growths of similar nature occurring in man and other animals (3, 4, 5), have had sufficient discussion in recent articles (6) to make comment unnecessary in this paper. The production of intumescences by means in which bacteria are not concerned has been noted recently by Smith (2) and Wolff (1).

In a paper by Dr. Erwin F. Smith (2, p. 167) it was suggested that osmotic relations between tumor and healthy tissues might offer an explanation for overgrowths. With a view to determining these osmotic relations the author has determined the freezing points of tumor and healthy tissues in different plants. It now appears that determinations referred to in a publication by Dr. Smith (3, p. 441) may be in error on account of the difficulty in obtaining the true freezing point of the tissues from the freezing point of the expressed juice.

The freezing point of tumor and healthy tissues taken from the same plants was obtained by expressing the juice with a Buchner hand press, and with an hydraulic press using 10 tons on a 2½ inch ram.

The freezing point of juices expressed from a tissue vary according to the treatment before expression and also according to the pressure applied (7, p. 94). Also, it appears that variation in the quantity of wood in the tissue prevents one from obtaining a uniform sample by pressure.

In table 1 are given the freezing points of juices expressed after freezing with solid CO_2 and grinding in a mortar while frozen dry. Stem tissue was taken from nodes immediately adjacent to the tumors. The tumor material was supplied by Dr. Smith's laboratory and was produced by inoculation with *Bacterium tumefaciens*.

TABLE 1. *Freezing points of juices from tumor and healthy tissues*

	Ricinus	
Leaf	− 0.793° C.	− 0.815° C.
Tumor	− 0.953 C.	− 0.746 C.
Stem	− 0.712 C.	− 0.590 C.
	Daisy	
Leaf	− 0.810° C.	
Tumor	− 1.170 C.	
Stem	− 0.783 C.	
	Beet	
Leaf	− 0.920° C.	
Tumor	− 0.970 C.	
Root	− 1.210 C.	

In Ricinus and daisy the tumor tissue yields an expressed juice with a greater freezing-point depression than that of adjacent normal tissue. But there is more woody tissue in the normal stem than in the tumor tissue of the Ricinus and daisy. The author does not regard these freezing points as the true values for the tissue. They are given for the purpose of showing the errors which may arise from the method of obtaining the freezing points of these tissues.

By using a thermocouple threaded through the tissue the freezing point can be obtained directly. The apparatus used in these determinations was the same as that reported in a previous paper (18). A piece of tissue of the same size in each case was threaded upon the thermal junction and cooled by ether evaporation to a desired point. Inoculation of the tissue was brought about by knocking it against the wall of the surrounding tube which was covered with frost. By this means the undercooling could be regulated quite accurately. When the undercooling is the same, the freezing points of the tumor tissue and of adjacent healthy tissue are nearly the same; in any case there is only a few hundredths of a degree difference, as shown in table 2.

TABLE 2. *Freezing points of tumor and healthy tissues obtained by thermocouple*

	Ricinus Undercooling	Freezing point
Tumor	− 1.49° C.	− 0.40° C.
Healthy stem	− 1.49 C.	− 0.41 C.
Tumor	− 2.20 C.	− 0.60 C.
Healthy stem	− 2.37 C.	− 0.64 C.
	Beet	
Tumor	− 2.92° C.	− 1.38° C.
Healthy root	− 2.90 C.	− 1.33 C.
Tumor	− 4.44 C.	− 1.74 C.
Healthy root	− 4.46 C.	− 1.72 C.

These values are free from errors arising in expression of the juice and represent the true freezing points of the tissue. They are of greater value than those made on expressed sap because small pieces of tissue can be taken immediately adjacent. Since such good checks were obtained, the author is inclined to believe that there is but little difference in the osmotic concentration in these particular cases.

Juices expressed from tumors produced by inoculation with *Bacterium tumefaciens* show a hydrogen-ion concentration consistently a little lower than that of juices expressed from healthy stem tissues taken from adjacent nodes, as shown in table 3.

TABLE 3. *H^+ concentrations of tumor and healthy tissues*

	Ricinus P_H	$C_H \times 10^6$
Tumor juice	5.822	1.51
Healthy stem juice	5.411	3.88
Leaf juice	5.580	2.63
Tumor juice	5.886	1.30
Healthy stem juice	5.817	1.52
Leaf juice	5.739	1.82
Tumor juice	5.62	2.40
Healthy stem juice	5.35	4.47
Leaf juice	5.48	3.30
	Beet	
Tumor juice	6.347	0.42
Healthy root juice	5.818	1.52

These tumors were in actively growing condition. The juice was expressed after grinding in a meat chopper but without freezing the tissue, since the hydrogen-ion concentration of a juice has been shown to be changed by the precipitation of the proteins on freezing the tissue. The determinations were made by the potentiometric method.

It may be suspected that the expressed juice will show a H^+ concentration different from that of the cell sap of the vacuoles owing to errors arising by expression. This may be the case in some tissues. However, the author has been able to dilute the juice from such tissues as tomato fruit to one fifth the original concentration without appreciably changing the H^+ concentration. The H^+ concentration within the uninjured cells can be estimated only in tissues which have natural indicators.

The concentration of the buffer salts present in the tissue will determine whether or not they are able to maintain the original P_H value on dilution. Precipitation of globulins on too great dilution of the juice may bring about H^+ changes. The H^+ concentration of a buffer solution depends (within fairly wide limits) upon the ratio of the buffer substances present, and not upon their total concentration. In obtaining the freezing-point depression, the total concentration of the expressed juice must be the same as that within the tissue to obtain a true value; for the H^+ concentration this is not necessarily the case.

Although the differences in H^+ concentration shown in the above table are small, they may be of relatively great importance for the activity of the respiratory enzymes associated with growth. The curve for the activity of catalase of plant origin at various H^+ concentrations is practically the same as that for catalase of animal origin given by Michaelis (8). From this curve it will be seen that the H^+ concentration shown by the plant tissue lies in a critical region for the activity of catalase. A change from P_H 5.52, the average value for healthy stem tissue of Ricinus, to P_H 5.78, the average value for tumor tissue, increases the catalase activity 25 percent at an acetate concentration of fiftieth normal. The activity of oxidase is increased also by a decrease in the H^+ concentration. In a paper cited above (7, pp. 98–101) the author gave indications of the decrease in H^+ concentration resulting from freezing of leaf tissue. Areas of Bryophyllum leaves which contain anthocyanin are changed from red to blue on freezing. This is not necessarily accompanied by death of the tissue.

It is shown from the work by Dr. Smith and others (9, p. 113) that *Bacterium tumefaciens* blues litmus milk and decreases the H^+ concentration of the culture medium. This it appears to do also in the tissue in which it grows.

Since catalase is destroyed at increasing rates with increase in the hydrogen-ion concentration, it is of interest to compare the catalase activity of two tissues of the same plant which were the same before overgrowth was induced by stimulation but which finally have different hydrogen-ion concentrations. For this purpose, Ricinus plants were inoculated with *B. tumefaciens* and when the tumors had become sufficiently large they were removed. Healthy stem tissue was taken from the same node. Fifty grams of tissue were ground in a mortar with crushed quartz and 25 cc. of phosphate buffer mixture C_H 2.7×10^{-8}, and made up to 500 cc. with distilled water. The Van Slyke apparatus for amino-acid determination was found convenient for catalase determination when used in a constant temperature room. Five cc. of hydrogen peroxide (Oakland 3 percent neutral) was run into the reaction chamber of the apparatus and washed down with 10 cc. of distilled water. After adjusting the level in the measuring pipette, 10 cc. of plant tissue dilution was run in from the side burette. The apparatus was shaken only fast enough to give a good mixing of the solution, and the rate of shaking was kept the same throughout. After 10 minutes the following amounts of oxygen were evolved at 30° C.

Tumor tissue	44.6 cc. O_2	42.3 cc. O_2
Healthy stem tissue	13.2 cc. O_2	13.3 cc. O_2

Bryophyllum leaves were inoculated with *B. tumefaciens* by injecting a suspension of the bacteria into the leaves by means of vacuum. This produced rather large tumored areas. Healthy leaf tissue which showed no tumor formation was taken from the same leaf. Three and three tenths grams of tumor and healthy tissue were diluted to 50 cc. after treating as before. Twenty-five cc. of these dilutions gave in the same time for tumor tissue 38.9 cc. O_2; and for healthy leaf, 2.4 cc. of oxygen.

Intumescences were also induced on Bryophyllum leaves by freezing the plants at a temperature of − 2° C. After about 15 minutes it was observed that frozen areas appeared over the surface of the leaf. The plants were removed from the cold chamber when the areas were about 5 mm. in diameter and placed in the greenhouse. After five days the areas had grown into small intumescences. While these were still actively growing, leaves were selected in which the tumors formed about half the area. It was found impossible to get a greater percentage of the area to grow out as intumescences because death occurred if more than about half the leaf surface was frozen. The tumor spots were so small and so intermingled with healthy tissue that a quantitative separation of the tumored and healthy areas could not be made without introducing great error.

Six grams of leaf tissue of which the tumored areas were estimated to represent half the tissue were taken and compared for catalase activity with 6 grams of normal tissue from a leaf of the same age and size from the same plant. The tissues were ground with quartz and excess $CaCO_3$ and made up to 200 cc. Twenty-five cc. of the dilution were taken with 5 cc. of the hydrogen peroxide. After 15 minutes the following amounts of oxygen were evolved:

Tumor 50 percent	3.1 cc. O_2
Healthy tissue	1.05 cc. O_2

Tissues of beet in which the overgrowths were produced by inoculation with *B. tumefaciens* gave on treatment in the exact manner given above for Ricinus tissue:

Tumor tissue	8.1 cc. O_2	8.9 cc. O_2
Healthy root tissue	5.6 cc. O_2	5.4 cc. O_2

It should be noted that the increase in catalase activity of the intumescences produced no Bryophyllum leaves by *Bacterium tumefaciens* is much greater than that of the intumescences produced by freezing.

The peroxidase and catalase activities in intumescences of cabbages induced by freezing are greater than those in normal tissues of the same leaf.

Since this decrease in H^+ concentration increases the catalase activity, it is interesting to note that the catalase activity is greatly decreased in mosaic leaves of tobacco which show greater H^+ concentration than healthy leaves. In this case also, the P_H values are but slightly different, yet it appears of great physiological importance for the growth of the mosaic-diseased cells.

The oxidase activity of tumor and healthy tissue was obtained on beets inoculated with *B. tumefaciens.* Tissue dilutions representing the same green weight of material were made up to C_H 3.6×10^{-7} and $n/60$ total salt concentration. Oxidase determinations were made in an oxidase apparatus which has been described in another place (10) and which is a modification of the simplified Bunzell (11) oxidase apparatus (12). After shaking for four hours at a constant temperature of 30.2° C., the following readings were given as the average of three determinations:

Oxidase activity of beet tissue

Tumor tissue	
Hydrochinone	3.24 cm. Hg.
Pyrogallol	4.14 cm. Hg.
Healthy tissue	
Hydrochinone	0.90 cm. Hg.
Pyrogallol	1.70 cm. Hg.

In this case it appears that the oxidase activity in the tumored areas is greatly increased. An increase in oxidase activity in tumored tissues has been reported in a number of articles (22).

In this connection it is interesting to note that the anthocyanin color is greatly increased in the tumored areas of Bryophyllum leaves induced by freezing, so that the intumescences stand out as red areas. The intumescences are produced mostly along the veinlets rather than in the vein islets. The deepest color of anthocyanin is located along the veinlets within the intumescences.

When Bryophyllum leaves are frozen in spots over a small percentage of the area, the frozen spots turn brown. One can predict from the depth of this brown color whether the area will die or will be stimulated to growth. As the percentage of the total area which is frozen increases, the depth of brown color in the spots decreases. When the whole leaf is frozen the color is uniformly distributed. If brown-spotted leaves are killed by freezing or by ether, the spotted areas maintain the deepest color, showing that the oxidizable substances have been partly removed from the surrounding tissues. It appears from this that there is an accumulation of the colored compounds within the frozen areas. This becomes very marked about 12 hours after freezing, as shown in the photograph (fig. 1). The greatest development of color occurs along the veinlets within the frozen areas (fig. 2). The frozen areas seem to have the property of taking up substances from the surrounding tissues which are converted within them and which accumulate as brownish colored compounds. Onslow (13) has given us data on the nature of such substances. Substances of the type of catechol occur in plants which brown on injury. Peroxidase activates the oxidation of these aromatic compounds, and the oxidized product is an organic peroxide.

It has been shown by Krassnosselsky (14) that an increased rate of respiration follows frost injury. The author so far has not been able to compare the respiratory rate of the frozen and normal areas. It would be desirable also to determine the oxygen acceptors in these spots. There is no immediate increase in the catalase activity of the areas frozen, nor does this increase until growth can be observed. Blackening indicates that there is an abnormally oxidized condition in the frozen areas.

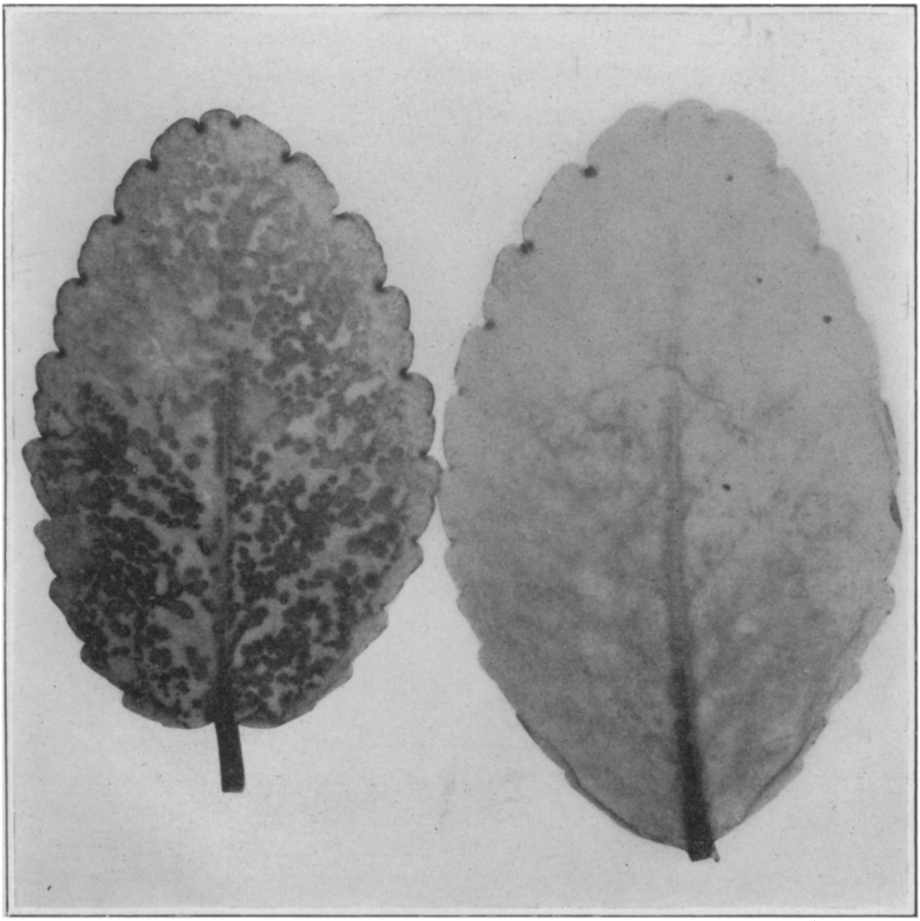

FIG. 1. Development of color in Bryophyllum leaves as a result of freezing. Notice the deep color in the small isolated frozen areas and the decrease of the color intensity with increasing size and frequency of the spots. The leaf with uniform color distribution was frozen throughout.

DISCUSSION

To the author it seems plausible that the hydrogen-ion concentration of the cell sap in the frozen areas may be decreased by removal of H^+ during the precipitation of proteins, which has been shown by the author to occur on freezing (7, p. 103). This decrease of H^+ concentration favors the activity of catalase and oxidase. The author has shown (7) that in hardened cabbage leaves there is no production of intumescences from frozen spots. The reason ascribed for this was that there was no permanent combination of protein and H^+ and hence no H^+ change or protein precipitation in this case. There may be attendant upon the protein precipitation, which could occur especially at the outer boundary of the protoplast, an increased permeability of the membranes for oxygen, so that the oxygen concentration within the cell is increased. The next attendant condition would be the formation of increased quantities of organic peroxide. The oxidation proceeds to the formation of colored compounds, finally producing substances such as purpurogallin from pyrogallol or melanins from tyrosine. The oxydo-reductases are prevented from normally reducing these colored compounds by the increased concentration of oxygen in the cells (15).

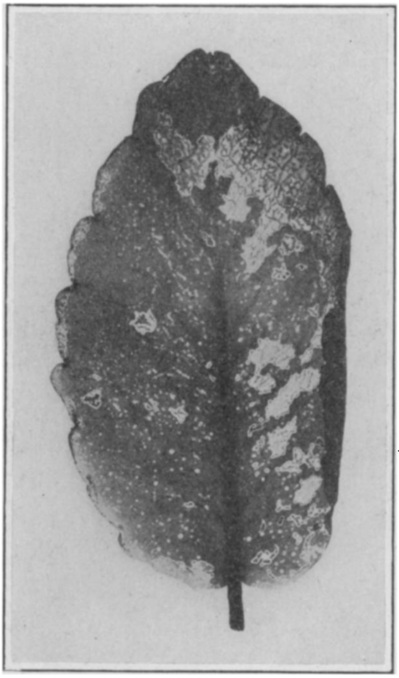

FIG. 2. Intumescences on Bryophyllum leaf induced by freezing. Notice occurrence of intumescences along the veinlets and the brown color along the veins in the light-colored areas. Light-colored areas are due to death of tissue because too great a percentage of the area was frozen. Local freezing occurred in these areas along the veins only, and brown color developed there. Subsequently the cells in the light-colored areas died. Renewed growth is not due to isolation, for three islets of normal tissue occur in the upper portion of the leaf and these show no renewed growth.

If the cell has been injured to such an extent as to be killed, all the oxygen acceptors are changed to the final state of equilibrium with the air. In case the cell recovers, an increased rate of oxidation has been established in it above the rate of oxidation in the cells in areas not frozen, owing to the greater concentration of organic peroxide and decreased H^+ concentration.

Child (16) has shown that dominance of growth is conditioned by a higher rate of metabolism in the growing region than in the surrounding area. When this increased rate is once established, it then opens the way for further growth at the expense of the surrounding tissue. In case too great a percentage of the leaf cells are frozen, there is no great concentration and transformation of the oxygen acceptors in any one area, as shown by little development of brown color. Hence there is little growth produced, because no one frozen area dominates a sufficiently large area of normal tissue which supplies it. This is actually the case. The most rapidly growing areas are small and somewhat scattered. Loeb (17) has suggested that the quantity of growth in *Bryophyllum calycinum* is conditioned by the quantity of some growth-promoting substances within the leaf. It is entirely possible that the usual sources of energy, the carbohydrates, are not the only substances concerned, but also that there are chromogenic substances of equal importance. Oxidase determinations indicate that there may be such substances produced or accumulated in plant tumors caused by *Bacterium tumefaciens*.

Loeb (21) also suggests that the dominance of a growing apex is due to the production within it of inhibiting substances which hold in check the neighboring buds. The difficulty with this assumption is that if the inhibiting substance is produced in the dominant apex it should depress growth there also since it would be in greatest concentration there. This ought to be a general objection to such assumptions. It seems to the author more plausible to assume that in correlation of growth the dominance of a growing area is conditioned not by the production of an inhibitor for the area around it, but by the removal from the surrounding area of growth-stimulating substances and their accumulation in the dominant area. These growth-stimulating substances, such as the chromogens of Palladin (23), are produced by all the cells and are diffusible. We have seen in a case cited above (*Bryophyllum*) how they accumulate in a frozen area and are associated with a renewal of cell growth.

In the case of inoculation of tissues with *Bacterium tumefaciens*, the presence of this bacterium favors the action of the respiratory enzymes by locally decreasing the H^+ concentration. This condition is continuous, and hence the growth process should be continuous. The bacteria in such tumor tissue evidently do not produce substances detrimental to cell multiplication, but may produce substances which favor oxidation within the tissue.

SUMMARY

It was found that the concentration of osmotic substances was the same in tumor and healthy tissues in Ricinus and beet when measured by the thermocouple method. Hence osmotic relations do not account for the tumor production by *Bacterium tumefaciens* in these cases.

Catalase, oxidase, and peroxidase activity is greater in tumor tissue than in adjacent healthy tissue either when the tumors are produced by inoculation with *B. tumefaciens* or by freezing.

Growth in frozen spots of Bryophyllum leaves is correlated with the accumulation within them from the surrounding tissue of substances of the nature of catechol, which are transformed by oxidation into colored compounds.

The H^+ concentration of tumors produced both by freezing and by bacterial inoculation is consistently less than that of adjacent healthy tissue.

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